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The sevenmaker gain-of-function mutation in p42 MAP kinase leads to enhanced signalling and reduced sensitivity to dual specificity phosphatase action

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Abstract A mammalian mutant MAP kinase, D319N ERK2, analogous to *Drosophila melanogaster* sevenmaker (rl^{sem}) gain-of-function mutation was shown to have an increased sensitivity to low levels of signalling in vivo. However, the mutation does not lead to an elevated basal kinase activity and still requires activation by MAP kinase kinase (MAPKK) as does wild type ERK2. This increased responsiveness seen in vivo is not due to an increased ability to phosphorylate substrates but appears to reflect a reduced sensitivity to a MAP kinase phosphatase CL100.

Key words: Sevenmaker; Protein kinase; Protein phosphatase; Signal transduction; Dual specificity

1. Introduction

Mitogen-activated protein (MAP) kinases (or extracellular signal-related kinases (ERKs)) are serine/threonine protein kinases that are activated in response to a wide variety of stimuli (for recent reviews see [1,2]). They play a pivotal role in the regulation of cellular proliferation and differentiation in response to extracellular signals acting on receptor tyrosine kinases (RTKs). MAP kinase substrates include $p90^{rsk}$ [3] cytosolic phospholipase A2 [4], MAPKAPK2 [5] as well as transcription factors c-Jun [6], Elk-1 [7] and c-Myc [8]. Upon activation MAP kinases translocate to the nucleus [9], thus phosphorylation of transcription factors by MAP kinase may provide a link between extracellular signalling and changes in cellular gene expression. However, while MAP kinase activation is an attractive mechanism for RTK mediated signal transduction in mammalian cells, there is little direct evidence that active MAP kinases mediate responses in mammalian cells.

Much of the genetic analysis of cell–cell interactions mediated by receptor tyrosine kinases has been carried out in *Drosophila melanogaster* and *Caenorhabditis elegans*, which have been shown to have signalling pathways with components homologous to the vertebrate MAP kinase pathway [10] and [11]. In *Drosophila* the correct specification of cell fates at the unsegmented termini of the embryo requires the RTK encoded by the gene *torso* (tor) [12,13]. The *sevenless* (*sev*) gene encodes another RTK which is required for the specification of the R7 photoreceptor cell fate in the *Drosophila* adult eye [14]. Binding of its ligand, the product of the *bride of sevenless* (*boss*) gene, which is expressed on the surface of the neighbouring R8 photoreceptor cell activates the sev tyrosine kinase in the R7 precursor cell [14,15].

In both of these *Drosophila* RTK pathways the immediate downstream event is activation of the $p21^{ras}$ homologue encoded by Dras1 in *Drosophila* [16,17]. Ras activation by RTKs occurs both in vertebrates and invertebrates via a SH3-SH2-SH3 adaptor protein (Drk/GRB-2) which acts as a link between activated RTKs and the Ras guanine nucleotide release factor Sos [18,19]. Sos is then able to activate Ras by increasing the rate of exchange of GTP for GDP [20,21]. In mammalian cells activated GTP-bound Ras translocates Raf to the plasma membrane where Raf may undergo further activation [22,23]. Activated Raf then leads to the phosphorylation and activation of MAP kinase kinase (MAPKK or MEK) [24,25] via phosphorylation of MAPKK on two serine residues [26]. MAPKK in turn activates MAP kinase by phosphorylation of both a threonine and a tyrosine residue [27,28].

Recent genetic analysis in Drosophila strongly implies that MAP kinase is an effector of the sevenless and torso signalling pathways [29]; the identification of loss-of-function mutations in the Map kinase *rolled* locus show that the *Drosophila* MAP kinase homologue, erk-A, is required downstream of Raf in the Sev signal transduction pathway [30]. Therefore MAP kinase activity appears to be necessary for signalling through the MAP kinase pathway. Furthermore, Brunner et al. [29] have identified a dominant gain-of-function mutation, known as sevenmaker, (rl^{sem}) in the *Drosophila* rl/MAP kinase gene by using a genetic screen selecting for mutants which had an activated sev signalling pathway in the absence of *boss* (the RTK ligand in the sevenless pathway). This mutation was shown to be a single amino acid substitution of the aspartate residue at position 334 into an asparagine residue. This residue is located at the C-terminal end of kinase domain XI in the catalytic domain of MAP kinase, a region which is conserved among all members of the MAP kinase family cloned to date. Sevenmaker activates not only the sev pathway but also the tor pathway and some developmental pathways controlled by the *Drosophila* EGF receptor homologue (DER) [31]. Thus it appears that MAP kinase acts at the end of a conserved signalling cascade used by multiple RTKs and that activated MAP kinase is necessary and sufficient for signal transduction in the pathway.

Gain-of-function mutations in MAP kinase would be of great value in studying the roles of MAP kinase in signal transduction in mammalian cells, in particular to determine whether activation of MAP kinase is sufficient for cell proliferation and differentiation. Furthermore analysis of the way the single point mutation leads to gain-of-function in MAP kinase may provide insight into the regulation of the enzyme. In order to understand the molecular basis for the gain-of-function and to study its effects on signal transduction we have therefore made

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the analogous sevenmaker mutation in the mammalian MAP kinase and have analysed the mechanisms which contribute to its increased activity.

2. Materials and methods

2.1. Expression plasmids

All constructs for COS-1 and NIH cell transfections with the exception of pSV2 β-gal and fos-lcf are based on pEXV3 [32] and with the exception on D319N ERK2myc have been described previously [24]. The pEXV3-D319N ERK2myc construct was made by mutating codon 319 of mouse ERK2myc to encode asparagine by site-directed mutagenesis with the Amersham M13 kit, sequenced to ensure that there were no unwanted mutations and subcloned into pEXV3. pFos Lcf and pSV2 β-galactosidase were the kind gifts of J.L. Bos (University of Utrecht, Utrecht, The Netherlands).

2.2. GST-fusion proteins

D319N and wild type murine ERK2 were cloned into pGEX2T, expression vectors for Gst-elk-1 and Gst-c-jun were generous gifts from R. Treisman (Imperial Cancer Research Fund, London, UK) and J.R. Woodgett (Ontario Cancer Institute, Toronto, Canada), respectively. Recombinant proteins were expressed as glutathione-*S*-transferase fusion proteins in *E. coli* and purified by affinity chromatography on glutathione-Sepharose. ERK2 was cleaved from glutathione-*S*-transferase with thrombin. Gst-elk-1 and Gst-c-Jun were used as intact fusion proteins bound to glutathione-Sepharose. Recombinant CL100 [33] protein was the kind gift of S.M. Keyse (Imperial Cancer Research Fund, Edinburgh, UK).

2.3. Transient transfection of COS cells

COS-1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (Gibco). Transfection, harvesting and Western blotting of cell lysates was carried out as previously described [24]. Immunoprecipitates were assayed for MBP kinase activity as previously described [33]. To confirm that equal amounts of ERK2 had been immunoprecipitated for each point, precipitated ERK2 was run out on a 10% gel and probed with the rabbit polyclonal antibody 122 against ERK2 [34].

2.4. NIH-3T3 cell transfections

For transient expression in NIH3T3 cells, cells were grown in DMEM containing 10% donor calf serum (Gibco). Transfections were carried out essentially as previously described [35]. Exponentially growing cells were seeded at a density of 2×10^5 cells per 100 mm plate and incubated overnight in growth medium. Then 25 μg of plasmid DNA was mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2 × BBS (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4 pH 6.95). The mixture was incubated for 10 min at room temperature. Calcium phosphate–DNA solution (1 ml) was added dropwise to the plate of cells and the mixture was swirled gently and incubated for 15–20 h at 37°C at 3% CO_2. The medium was removed and the cells were rinsed twice with Tris-saline, 5% donor calf serum, 20 mM HEPES pH 7.1, refed with DMEM + 5% DCS and incubated for 5 h. The medium was then changed to DMEM + 0.5% DCS and the cells were incubated for a further 48 h at 37°C, 10% CO_2. Cells were lysed using the Promega Luciferase assay system lysis buffer. Lysates were assayed for β-gal expression [36], equilibrated for their β-gal levels to 0.5 U/ml then assayed for luciferase levels using the Promega Luciferase assay system.

2.5. Assay of MAP kinase activity using recombinant proteins

Inactive recombinant MAP kinase was activated by incubation with constitutively active E217/E221 MAPKK [37]. Both enzymes were diluted in 100 mM Tris-HCl pH 7.5, 0.2 mM Na-EGTA, 0.1% (v/v) 2-mercaptoethanol (Basic buffer) to give a final assay concentration of 0.18 mg/ml, MAP kinase, 0.01 mg/ml MAPKK. 20 μl of Mg-ATP (0.8 mM ATP, 40 mM Mg-acetate) was added to 60 μl of the diluted MAP kinase/MAPKK mix to start the reaction. After 20 min at 30°C the reaction was terminated by placing on ice and diluting 1:5 with BSA buffer (50 M Tris, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 1 mg/ml BSA). The activated MAP kinase was either used immediately or frozen at 20°C for up to 1 week. For experiments with CL100, activated MAP kinase was incubated in BSA buffer with purified CL100 protein (diluted in 50 mM Tris, pH 7.5, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 0.04% Brij 35) for 10 min at 30°C. MAP kinase activity was then assayed by adding substrate kinase assay buffer (50 mM Tris 7.5, 0.1 mM EGTA, 0.4 mg/ml MBP or 1–2 mg/ml Gst-c-jun or Gst-elk-1, 0.5 mM Mg-acetate, 0.125 mM ATP and 63 μCi/ml ^{32}P) and incubating for 10 min at 30°C. Incorporation of phosphate into MBP was determined as described above. Incorporation of phosphate into Gst-c-jun or Gst-elk-1 was measured as previously described [37].

3. Results

3.1. Gain-of-function of D319N ERK2 is not due to constitutive activation

One explanation for the gain-of-function of the sevenmaker MAP kinase allele would be constitutive activation of the kinase in the absence of activation by MAPKK. We therefore transiently expressed epitope tagged D319N ERK2 in COS-1 cells and examined the kinase activity of D319N ERK2 in the absence of growth factor stimulation. Western blots of transiently transfected COS cells showed that D319N ERK2 ran with an increased mobility relative to wild type ERK2 in unstimulated cells and upon EGF stimulation both wild type and D319N ERK2 shifted to slower migrating phosphorylated forms [34]. The shift of D319N was slightly more pronounced than with wild type ERK2 (Fig. 1A). Kinase assays of immunoprecipitated D319N ERK2 showed that, in the absence of growth factor stimulation, its basal kinase activity was no higher than wild type ERK2. EGF stimulated wild type ERK2 kinase activity increased by 6-fold over that of unstimulated cells and D319N showed an 11-fold increase (Fig. 1B) which

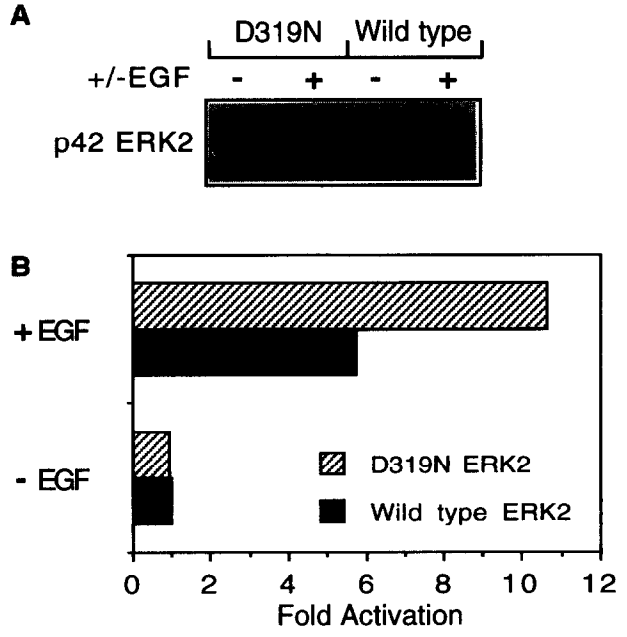

Fig. 1. Phosphorylation and activation of D319N ERK2. Cell lysates were prepared from COS-1 cells transfected (as described in section 2) with pEXV3-ERK2myc or pEXV3-D319N ERK2myc then stimulated with no addition (−) or 2 nM EGF for 10 min prior to cell harvesting (+). (A) Transfected ERK2 was detected in cell lysates by western blotting as described in section 2. Data essentially identical to these have been obtained in at six separate experiments. (B) ERK2myc was immunoprecipitated from cell lysates and the MBP kinase activity of the immunoprecipitates determined. Data shown are the average of six separate experiments.

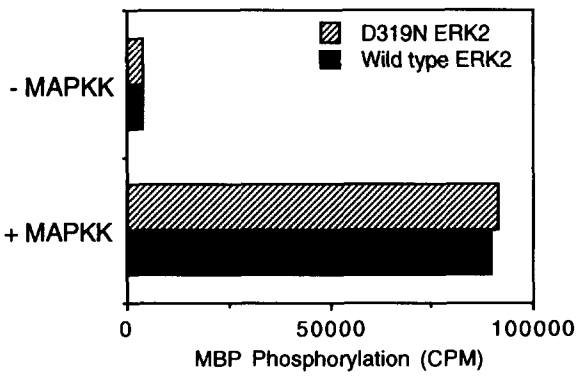

Fig. 2. Kinase assay of recombinant D319N ERK2. D319N ERK2 or wild type ERK2 were incubated in the absence (− MAPKK) or presence (+MAPKK) of MAPKK as described in section 2. They were then assayed for MBP kinase activity. Results above are the average of duplicate determinations. Similar results have been obtained in at least 3 separate experiments.

was also reflected in the more pronounced shift (Fig. 1A). To further confirm that D319N ERK2 does not have an elevated basal kinase activity it was expressed as a GST fusion protein in bacteria. The basal activity of recombinant D319N ERK2 was no higher than wild type and activation of both mutant and wild type MAP kinase require phosphorylation by MAPKK. Additionally, under these conditions, when phosphorylated by MAPKK the levels of kinase activity for recombinant D319N and wild type ERK2 are the same (Fig. 2). These results with both transient expression in mammalian cells and purified recombinant proteins demonstrate that D319N ERK2 does not have elevated basal kinase activity and still responds to growth factor stimulation. Thus the gain-of-function resulting from the mutation is not a consequence of constitutive activation of the kinase.

3.2. *D319N ERK2 is more responsive than wild type ERK2 to low levels of signalling*

Since D319N ERK2 does not show constitutive activation of kinase activity we considered the possibility that its gain-of-function results from an increase in its response to signalling. Such an idea is attractive given that sevenmaker causes a phenotype that corresponds to those of weak gain-of-function mutants in *sev* and *tor* RTKs [29]. We therefore decided to turn to another system to determine whether D319N ERK2 is better able to respond to signals than wild type ERK2. It has been previously demonstrated that transfection with oncogenic H-Ras or Raf leads to increased expression from the c-fos promoter in transient expression systems [38]. We therefore investigated whether cotransfection of ERK2 expression plasmids had an effect on c-fos promoter expression stimulated by oncogenic Ras or Raf. When transfected alone, wild type ERK2 or D319N ERK2 had no effect on c-fos driven luciferase activity. A striking difference was seen when ERK2 plasmids were cotransfected with the oncogenic Raf construct. Oncogenic Raf on its own produced a small degree of c-fos promoter activation but when cotransfected with D319N ERK2 this activation was potentiated 12-fold. In contrast, cotransfection with wild type ERK2 produced no potentiation (Fig. 3). The level of transcription induced by oncogenic H-Ras increased 4-fold when wild type ERK2 was cotransfected. D319N ERK2 increased the level of H-Ras induced transcription by 2.5-fold, a somewhat lesser degree than did wild type. One explanation for this may be that the signal generated by H-Ras + wild type ERK2 is maximal in this system and that D319N under circumstances of strong activation is a less active kinase than is wild type. In this system Raf is a poorer activator of the fos promoter than Ras and thus, with the weaker activation a marked difference is seen between the properties of the wild type ERK2 and the gain-of-function mutant D319N.

One possibility for the increased ability of D319N ERK2 to activate the c-fos promoter is that its substrate specificity is

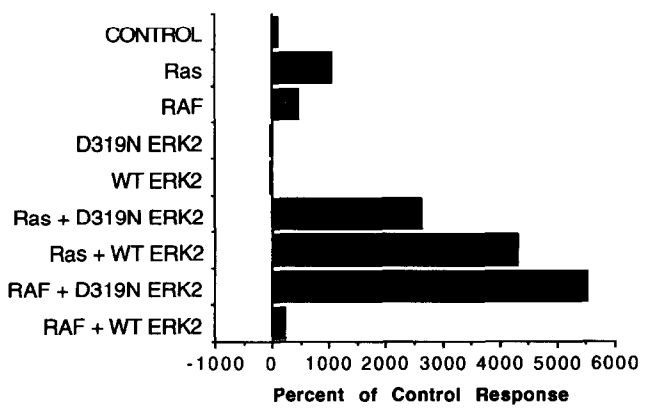

Fig. 3. Potentiation of oncogenic Raf signal by D319N ERK2. Cell lysates were prepared from NIH-3T3 cells transfected as described in section 2. Lysates were assayed for luciferase activity. Data shown above are the average of duplicate determinations expressed as a percent of activity with the control plasmid only.

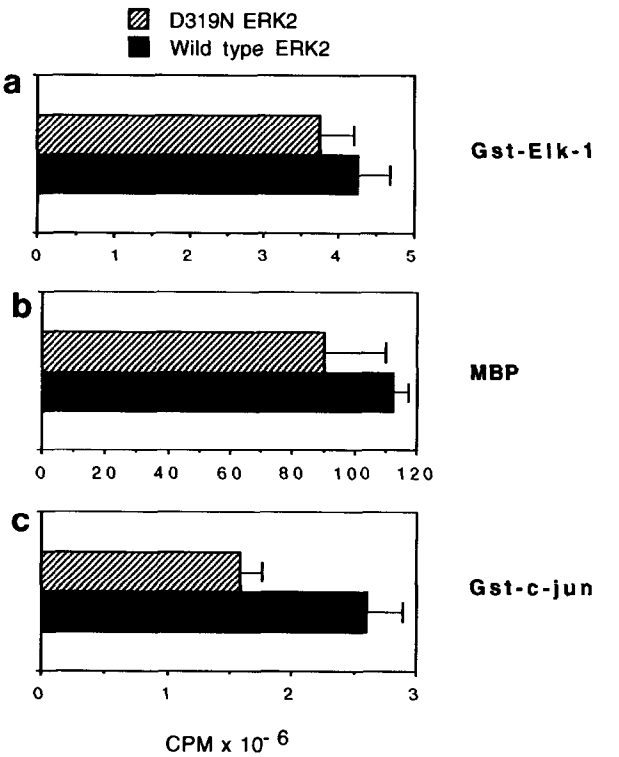

Fig. 4. Phosphorylation of MAP kinase substrates by D319N ERK2. Recombinant D319N and wildtype ERK2 were activated and tested (as described in section 2) for their ability to phosphorylate (A) Gst-Elk-1, (B) MBP and (C) Gst-c-jun in vitro. Results shown are the average of triplicate determinations. Error bars represent standard deviation.

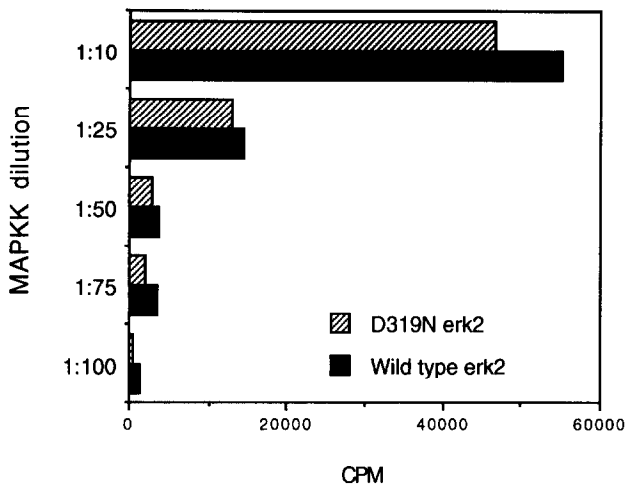

Fig. 5. Activation of D319N ERK2 by MAPKK. Recombinant D319N and wildtype ERK2 were activated with varying concentrations of MAPKK as indicated above. The activated ERK2 was tested for its ability to phosphorylate MBP in vitro (as described in section 2). Results shown are the average of duplicate determinations. Duplicates were within 10% of the mean.

altered or increased. Recently Marais et al. [7] have shown that elk-1, which complexes with SRF transcription factors to regulate c-fos expression, is activated by MAP kinase phosphorylation. We therefore examined whether active D319N ERK2 was more able than the wild type ERK2 to phosphorylate elk-1 in vitro. Under the conditions tested, there was very little difference in the levels of phosphorylation of elk-1 or MBP by active D319N ERK2 or wild type ERK2 (Fig. 4a,b). Similarly, another putative transcription factor substrate, c-jun was also phosphorylated no better by D319N than by wild type ERK2 (Fig. 4c). In fact, all three substrates tested appeared to be poorer substrates for the D319N ERK2 than for wild type ERK2 which implies that a higher kinase activity toward these substrates is not responsible for the gain-of-function properties of D319N ERK2.

The increased responsiveness of D319N ERK2 could be a result of its being activated at lower levels of MAPKK than the wild type ERK2. To test this hypothesis recombinant ERK2 and D319N ERK2 were activated with decreasing amounts of constitutively active MAPKK in vitro (Fig. 5). Five concentrations of MAPKK tested did not activate D319N ERK2 to a higher level than wild type ERK2, indicating that the difference is probably not due to D319N ERK2 being activated by lower levels of active MAPKK.

3.3. D319N ERK2 shows reduced sensitivity to MAP kinase phosphatase

Since our experiments revealed no constitutive kinase activity of D319N ERK2, we explored the possibility that D319N ERK2 is less sensitive to negative regulation. Recently it has become clear that there is a class of phosphatases which is specific for MAP kinases [39–41]. The archetype of these phosphatases is CL100/3CH134, whose transcription is inducible with oxidative stress or growth factors [42,43]. Therefore, we chose to look at the sensitivity of D319N ERK2 to the action of the phosphatase CL100. Recombinant ERK2 and D319N ERK2 were activated with MAPKK and then treated with varying concentrations of CL100 and assayed for kinase activity (Fig. 6). Both ERK2 and D319N ERK2 were inactivated in a dose dependent fashion by CL100. However, D319N was significantly less susceptible to inactivation. At 0.5 μg/ml CL100 the D319N ERK2 showed only 53% inactivation compared with 80% for the wild type.

4. Discussion

We have shown that the D319N sevenmaker mutation in ERK2 does not result in a constitutively active kinase. To become activated, mutant ERK2 from COS cell transfections or produced in bacteria, requires phosphorylation by MAPKK. However in vivo, D319N is more responsive to low levels of signalling than is wild type ERK2. Results from cotransfections of NIH-3T3 cells show that D319N ERK2 enhances the effect of oncogenic Raf while wild type ERK2 has no effect. This increased sensitivity to signalling does not appear to be a result of increased sensitivity to MAPKK or to an increased ability of D319N to phosphorylate substrate. However, the gain-of-function mutation D319N is more resistant to the action of a MAP kinase phosphatase CL100.

Although this mutation was originally isolated from Drosophila which lacked *boss*, the *sev* ligand, it is not surprising that the gain-of-function is not through constitutive kinase activity. The sevenmaker mutation was isolated as a viable mutation whereas expression of constitutive mutants of Draf and Dras expressed under the control of the heat shock promoter are lethal. With the characterization of the rolled/MAP kinase locus of Drosophila, the sem mutation/activated MAP kinase has been shown to be involved in multiple signalling pathways including sev, torso and DER. It therefore might be expected that a constitutively activated MAP kinase involved in these pathways would lead to a lethal mutation, which would not have been seen in the screen for sevenmaker. Although the difference in sensitivity to phosphatase inactivation is small, it is clear from the genetics of the R7 signalling system [10] that

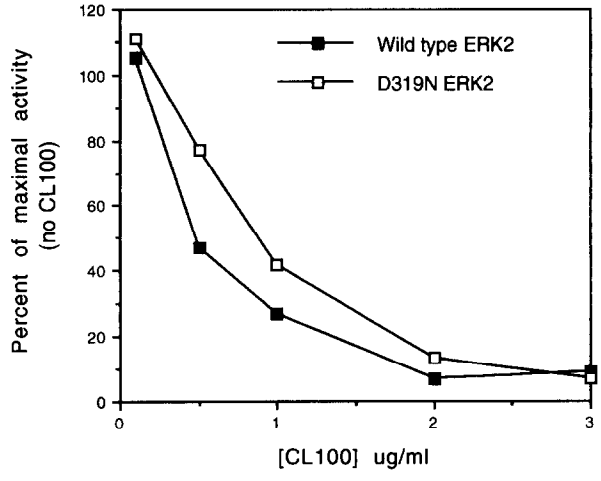

Fig. 6. Reduced sensitivity of D319N ERK2 to inactivation by CL100. Activated recombinant D319N and wild type ERK2 were treated with varying concentrations of recombinant CL100 (as described in section 2) and assayed for their ability to phosphorylate MBP in vitro. Results shown above are the average of triplicate determinations. Error bars represent standard deviation. Similar results have been obtained in three separate experiments.

small changes in the dosage of components of the signalling pathway can have pronounced effects. Thus a 2-fold difference in sensitivity to phosphatase action could well lead to altered signalling. It is also possible that reduced sensitivity to phosphatase action is only part of the explanation for why D319N ERK2 is more active than wild type ERK. For example there may be other substrates for MAP kinase which are differentially phosphorylated by the sevenmaker mutant which potentiate the activation of sevenmaker ERK2.

Although this gain-of-function mutation is not constitutively active and therefore does not eliminate the need for upstream signals, it may provide insight into downstream events of the MAP kinase pathway. In particular, D319N ERK2 may aid in the study of MAP kinase specific phosphatases and the role which the inactivation of MAP kinase plays in cellular growth and differentiation.

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